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Pollen Profilin Function Depends on Interaction with Proline-Rich Motifs

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The actin binding protein profilin has dramatic effects on actin polymerization in vitro and in living cells. Plants have large multigene families encoding profilins, and many cells or tissues can express multiple profilin isoforms. Recently, we characterized several profilin isoforms from maize pollen for their ability to alter cytoarchitecture when microinjected into living plant cells and for their association with poly-L-proline and monomeric actin from maize pollen. In this study, we characterize a new profilin isoform from maize, which has been designated ZmPRO4, that is expressed predominantly in endosperm but is also found at low levels in all tissues examined, including mature and germinated pollen. The affinity of ZmPRO4 for monomeric actin, which was measured by two independent methods, is similar to that of the three profilin isoforms previously identified in pollen. In contrast, the affinity of ZmPRO4 for poly-L-proline is nearly twofold higher than that of native pollen profilin and the other recombinant profilin isoforms. When ZmPRO4 was microinjected into plant cells, the effect on actin-dependent nuclear position was significantly more rapid than that of another pollen profilin isoform, ZmPRO1. A gain-of-function mutant (ZmPRO1-Y6F) was created and found to enhance poly-L-proline binding activity and to disrupt cytoarchitecture as effectively as ZmPRO4. In this study, we demonstrate that profilin isoforms expressed in a single cell can have different effects on actin in living cells and that the poly-L-proline binding function of profilin may have important consequences for the regulation of actin cytoskeletal dynamics in plant cells.

INTRODUCTION

The male gametes of plants are delivered to the ovule by a unique mode of motility that is dependent on cellular morphogenesis of the pollen grain (reviewed in Bedinger and Edgerton, 1989; Mascarenhas, 1993; Taylor and Hepler, 1997). Mature pollen is a haploid multicellular structure consisting of a large vegetative cell and one generative cell or two sperm cells. The sperm are derived from a mitotic division of the generative cell that occurs before pollen maturation or during pollen tube extension. When a pollen grain lands on a receptive stigma, the vegetative cell forms a tip-growing protuberance, the pollen tube, that extends down the transmitting tissue of the style toward the ovule. Remarkably, the growth rate of the maize pollen tube can exceed 1 cm/hr (Bedinger, 1992). The pollen tube delivers the sperm cells to the embryo sac, where they are deposited to fuse with the egg cell and central cell.

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Both pollen germination and pollen tube elongation are dependent on the actin cytoskeleton, as determined through studies with cytochalasins (reviewed in Pierson and Cresti, 1992; Taylor and Hepler, 1997). The actin cytoskeleton in pollen is highly dynamic, and dramatic rearrangements occur during pollen germination. Actin microfilaments focus on the germination aperture(s) before generation of the pollen tube and become arranged in long axial bundles that serve as tracks for the streaming of vesicles and organelles during pollen tube elongation. Because actin is important for the maintenance of tip growth, understanding how actin cytoskeleton organization and redistribution are regulated is fundamental to understanding how morphogenesis and polar growth of the pollen tube are coordinated.

Reorganization of the actin cytoskeleton in eukaryotic cells is regulated by a variety of actin binding proteins that modulate the behavior of both filamentous actin (F-actin) and globular actin (G-actin) (Hartwig and Kwiatkowski, 1991; Hatano, 1994). Profilin is a G-actin binding protein that was first discovered in a 1:1 complex with actin in extracts from spleen (Carlsson et al., 1977). It also interacts with poly-L-proline (PLP) and polyphosphoinositides (Sun et al., 1995; Schlüter et al., 1997). Profilin has been found in organisms from all eukaryotic kingdoms and plays a significant role in

regulating the dynamics of the actin cytoskeleton (reviewed in Sun et al., 1995; Schlüter et al., 1997; Staiger et al., 1997). There are, however, conflicting data concerning the activities of profilin in living cells. For example, microinjection of profilin into plant or animal cells caused a reduction in the F-actin levels (Cao et al., 1992; Staiger et al., 1994). In contrast, when profilin was stably overexpressed in mammalian cells, the level of F-actin increased without an increase in total actin (Finkel et al., 1994). The biochemical basis for this apparent paradox has been investigated, and it was found that profilin could "desequester" actin from a pool of sequestered actin monomers if the barbed ends of the filaments were not capped (Pantaloni and Carlier, 1993).

Profilin was first identified in plants as a birch pollen allergen (Valenta et al., 1991) and has since been shown to be present as a multigene family in many plant species (reviewed in Staiger et al., 1997). Multiple profilin isoforms could be required for tissue-specific or temporal regulation of profilin expression, as has been suggested for multiple actin genes in plants (Meagher, 1991). In fact, profilin isoforms display tissue-specific expression patterns in maize (Staiger et al., 1993) and Arabidopsis (Christensen et al., 1996; Huang et al., 1996). Multiple isoforms of profilin are expressed in the pollen of many species, which raises the question whether these are functionally redundant or whether they perform unique functions. The intracellular distribution of profilin in pollen from several different plant species has been investigated by using immunocytochemical analyses (Grote et al., 1993, 1995; Hess et al., 1995; Vidali and Hepler, 1997) and the microinjection of fluorescent profilin analogs (Vidali and Hepler, 1997). The results of these studies indicate that profilin is distributed uniformly in the vegetative cell cytoplasm and pollen tube, and suggest that the multiple isoforms are functionally similar. However, the inability to discriminate among multiple isoforms and/or to detect profilin in complexes with other proteins could mask any differences in isoform-specific localization patterns.

At least three profilin isoforms are expressed in maize pollen: *ZmPRO1*, *ZmPRO2*, and *ZmPRO3* (Staiger et al., 1993). The recombinant isoforms and native maize pollen profilin have been compared for G-actin binding and PLP binding activities (Gibbon et al., 1997). Furthermore, their relative ability to perturb cytoarchitecture when microinjected into live plant cells has also been analyzed. All of the recombinant pollen profilin isoforms and native pollen profilin have similar activities in vitro or in vivo and generally cannot be distinguished from each other by statistical analyses (Gibbon et al., 1997).

In this report, we characterize a fourth profilin from maize, ZmPRO4. This new profilin isoform is expressed predominantly in endosperm tissue but is also found in mature and germinated pollen. Recombinant ZmPRO4 was characterized for PLP binding, binding to pollen G-actin, and its ability to alter cellular architecture. When compared with other maize profilin isoforms, ZmPRO4 has a significantly greater affinity for PLP and more rapidly disrupts cellular architecture in *Tradescantia virginiana* stamen hair cells. These effects were mimicked by creating a gain-of-function mutant for another profilin isoform (ZmPRO1-Y6F), which had enhanced PLP binding and the ability to disrupt cytoarchitecture when compared with the wild-type protein. These results imply that profilin function can be modulated by interactions with proline-rich proteins and that individual profilin isoforms are suited to specific tasks in pollen.

RESULTS

ZmPRO4 Encodes a Plant Profilin

The full cDNA insert from clone 5C06B09 (Shen et al., 1994) is 830 nucleotides long and contains a major open reading frame of 396 nucleotides, a consensus translation start site, and a poly(A) tail of 35 nucleotides. The 5′ and 3′ untranslated regions are 115 and 285 nucleotides, respectively. Conceptual translation of the open reading frame predicted a polypeptide 131 amino acids long, as shown in Figure 1, with a calculated M_r of 14,100 and estimated pI of 4.43. The amino acid sequence of ZmPRO4 is 73 to 82% identical to the maize pollen profilin sequences, and the highest identity (82%) is found with the maize profilin ZmPRO1. The ZmPRO4 sequence shares 31 to 46% identity with vertebrate and lower eukaryotic profilins. The most highly conserved residues among all of the profilin sequences are those implicated in PLP binding (marked by asterisks in Figure 1), and all of these residues are present and conserved in ZmPRO4. Also indicated in Figure 1 are residues conserved among the plant profilin sequences that are thought to be involved in polyphosphoinositide binding (Drøbak et al., 1994) and actin binding (Thorn et al., 1997).

RNA Gel Blot and Reverse Transcriptase–Polymerase Chain Reaction Analysis of *ZmPRO4* Expression

Total RNA from a number of maize tissues was separated on glyoxal– or formaldehyde–agarose gels, transferred to nylon membranes, and probed at high stringency with gene-specific and coding region probes from *ZmPRO4.* Figure 2 shows that the gene-specific probe hybridized with a transcript ~900 nucleotides long that is expressed predominantly in endosperm tissue (Figure 2A). The same gene-specific probe hybridized with only one band for each of the digests on a DNA gel blot, demonstrating that this is a unique member of the maize profilin gene family (data not shown). The coding region probe detected a transcript in all tissues examined, including mature and germinated pollen, but was most abundant in endosperm tissue (Figure 2B). Similar expression patterns were observed with a filter from a formaldehyde–agarose gel that was probed with the gene-specific probe (data not shown).

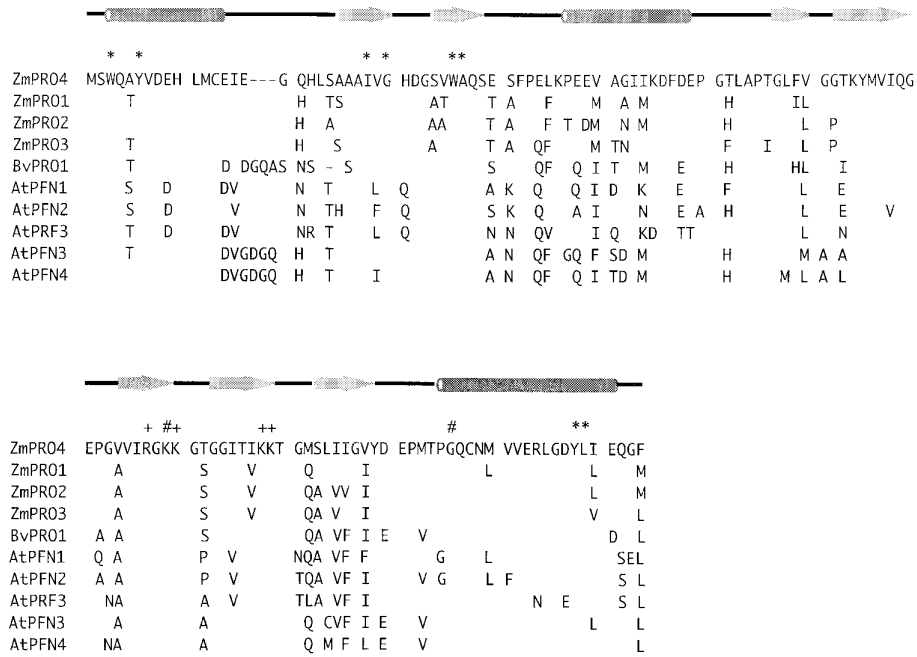

Figure 1. Comparison of the Deduced Amino Acid Sequence for ZmPRO4 with Selected Plant Profilins.

Secondary structure elements, with arrows indicating α-helical regions and cylinders representing β strands, are diagrammed above the primary sequence alignment and are positioned according to crystallographic data from birch pollen profilin (Fedorov et al., 1997). The positions of highly conserved residues, which are present in the majority of eukaryotic profilins, are indicated as follows: asterisk, PLP binding; (+), polyphosphoinositide binding; and pound sign, actin binding (see text for details). The sequences used for the alignment, their sources, and GenBank accession numbers or original citations are as follows: *ZmPRO4*, *Zea mays* profilin 4, AF032370 (this study); *ZmPRO1*, maize profilin 1, X73279; *ZmPRO2*, maize profilin 2, X73280; *ZmPRO3*, maize profilin 3, X73281; *BvPRO1*, *Betula verrucosa* profilin, M65179; *AtPFN1*, *Arabidopsis thaliana* profilin 1, U43325 (see also Huang et al., 1996); *AtPFN2*, Arabidopsis profilin 2, U43326 (see also Huang et al., 1996); *AtPRF3*, Arabidopsis profilin 3 (Huang et al., 1996); *AtPFN3*, Arabidopsis profilin 3, U43323 (see also Huang et al., 1996); and *AtPFN4*, Arabidopsis profilin 4, U43324. Multiple sequence alignments were created using the PileUp program from the Genetics Computer Group (Madison, WI; Devereux et al., 1984). Only residues that differ between ZmPRO4 and the aligned sequences are shown. Dashes indicate where gaps have been introduced to optimize alignment.

To confirm that *ZmPRO4* was expressed in mature pollen, a first-strand cDNA population generated from ungerminated pollen mRNA was used as a template for polymerase chain reaction (PCR) amplification with gene-specific primers for *ZmPRO1* and *ZmPRO4* (Figure 2C). In each case, a product of the expected size was obtained. The cDNA clones for *ZmPRO1* and *ZmPRO4* were included as controls. Serial dilution of the cDNA used in the reactions revealed that *ZmPRO4* was qualitatively less abundant than *ZmPRO1.* For *ZmPRO4*, little or no product was amplified at a dilution of 1:10,000, whereas a product was still readily detectable for *ZmPRO1* at the same dilution. The identity of the PCR products was confirmed by digestion with SacI and SstI, which have restriction sites only in the 3′ untranslated region of *ZmPRO4.* Only the *ZmPRO4* product was digested, and the restriction fragments were of the expected length (208 and 77 bp), confirming that the *ZmPRO4* gene-specific product was indeed from the 3′ untranslated region of the *ZmPRO4* message.

Profilin Is Present in Endosperm Tissue

Because the *ZmPRO4* cDNA was isolated from a library made from endosperm (Shen et al., 1994), we wanted to confirm that profilin protein could be isolated from developing endosperm tissue. Therefore, as shown in Figure 3, proteins purified with PLP–Sepharose from extracts of endosperm collected at 12, 16, and 20 days after pollination were analyzed by PAGE (Figure 3A, lanes 1 to 3) and protein gel immunoblots (Figure 3B, lanes 1 to 3). Recombinant ZmPRO4 that was expressed in *Escherichia coli* and purified by PLP–Sepharose affinity chromatography was included as a control (Figures 3A and 3B, lanes 4 and 5). The polyclonal profilin

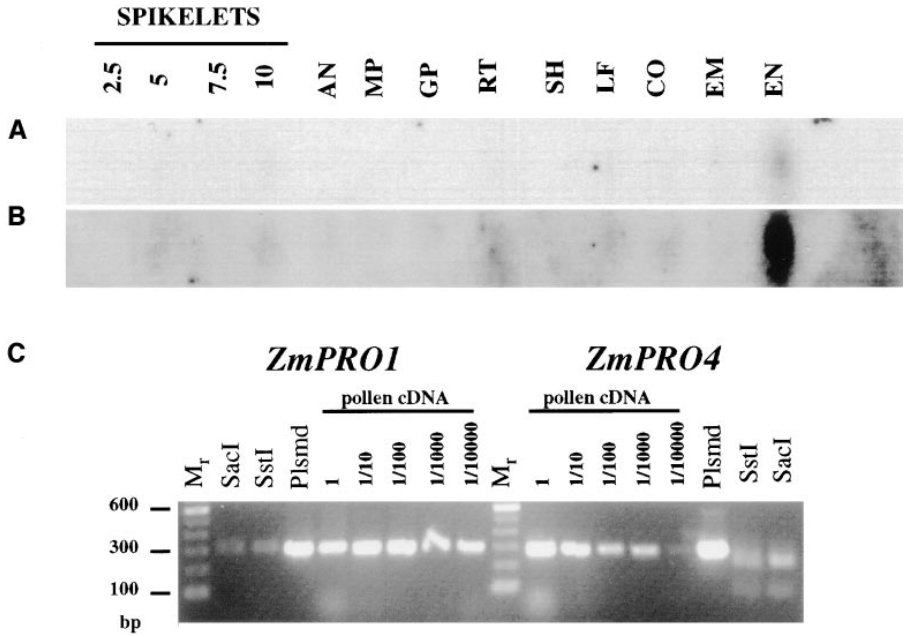

Figure 2. RNA Gel Blot Analysis of the *ZmPRO4* Transcript in Various Maize Tissues and Reverse Transcription–PCR Detection of Profilin Transcripts in Pollen.

The tissues were male reproductive spikelets at different developmental stages (premeiosis [2.5 mm], meiosis [5 mm], microspore [7.5 mm], and maturing pollen grains [10 mm]), anthers dissected from mature spikelets (AN), mature pollen (MP), in vitro–germinated pollen (GP), primary root tips (RT), etiolated shoots (SH), mature leaf (LF), cob (CO), embryo (EM), and endosperm (EN).

(A) *ZmPRO4* gene–specific probe followed by high-stringency washes. The *ZmPRO4* transcript is present predominantly in the endosperm tissue.

(B) *ZmPRO4* coding region probe followed by high-stringency washes. The transcript is most abundant in endosperm tissue but is also present in all other tissue samples.

(C) Reverse transcription–PCR amplification of maize pollen mRNA by using gene-specific primer pairs for *ZmPRO1* and *ZmPRO4.* Both primer pairs amplified products of the expected length from control plasmids carrying cDNA clones for *ZmPRO1* and *ZmPRO4* (Plsmd). The products obtained using pollen cDNA were the same length as the products from the control plasmids. Serial dilution of the cDNA template showed that the *ZmPRO4* transcript was less abundant than that of *ZmPRO1.* The PCR product for *ZmPRO1* was not digested by SacI or SstI (outermost left lanes), whereas that for *ZmPRO4* was digested and the fragments were of the expected lengths (outermost right lanes). The molecular mass markers are 100-bp ladders (indicated at left).

antibody recognizes an endosperm protein of the appropriate size (M_r of 14,200), thus demonstrating that profilin protein is present in endosperm tissue at this developmental stage (Figure 3B, lanes 1 to 3). When different extraction and sample preparation conditions are used, a minor 21-kD protein copurifies with the 14-kD profilin from endosperm and is recognized by the profilin antibody (data not shown). The possibility that this 21-kD species represents a novel profilin isoform or a post-translational modification will be the subject of future investigation.

ZmPRO4 Is Efficient at Perturbing Actin-Dependent Nuclear Position in Live Cells

To test the effect of actin binding proteins on F-actin and cytoplasmic organization in the complex environment of the living cell, we have developed the following model system to quantify actin-dependent nuclear positioning (Gibbon et al., 1997; Ren et al., 1997). The introduction of excess profilin into *T. virginiana* stamen hair cells by pressure microinjection results in a rapid and dramatic reduction in the number of transvacuolar cytoplasmic strands, disrupts the F-actin cytoskeleton, and results in the displacement of the nucleus from a central position (Staiger et al., 1994). When several experimental parameters are held constant (size and position of the cell within the chain of cells that comprise the stamen hair, protein concentration in the needle, and volume of injectate), different profilin isoforms can be compared directly by measuring the time required for the nucleus to move from the starting position marked by its perimeter (Gibbon et al., 1997). For the experiments reported here, 5 to 6 pL, at needle concentrations ranging from 50 to 200 μM profilin, equivalent to ~10% of the cytoplasmic volume was

injected. Microinjection of 100 μM profilin in the micropipette resulted in an estimated increase of the cytoplasmic profilin concentration from ~6 μM (Staiger et al., 1994) to ~16 μM. At least 30 cells were examined for each treatment, and the data are plotted as the mean time required for nuclear displacement, as shown in Figure 4. Control cells that were not injected rarely underwent nuclear migration sufficient to be defined as displacement and therefore had an average nuclear displacement time of 19.5 ± 0.2 min (mean ±SE; $n = 31$; Figure 4B). Microinjection of the buffer alone had an average nuclear displacement time of 18.0 ± 0.7 min (Gibbon et al., 1997) or 17.8 ± 1.0 min (Ren et al., 1997). Cells microinjected with bovine γ-globulin, to rule out effects of introducing foreign protein into the cytoplasm, had an average nuclear displacement time of 19.2 ± 0.4 min ($n = 31$). The average displacement times for uninjected, buffer-injected, and γ-globulin–injected controls were not significantly different from each other by the *t* test ($P > 0.05$).

Nuclear displacement was dependent on the profilin concentration for both ZmPRO1 and ZmPRO4 (Figure 4A), and at each concentration tested, ZmPRO4 was substantially faster than ZmPRO1. Microinjection of 100 μM ZmPRO1 caused nuclear displacement in 6.6 ± 0.8 min ($n = 32$), and 100 μM ZmPRO4 caused nuclear displacement with an average time of 4.1 ± 0.6 min ($n = 35$). These values were significantly different by the *t* test ($P = 0.01$). The difference was also observed when cells were microinjected with 200 μM profilin; ZmPRO1 averaged 4.5 ± 0.5 min ($n = 32$), whereas ZmPRO4 averaged 2.7 ± 0.3 min ($n = 33$). These values were also significantly different by the two-tailed *t* test ($P = 0.004$). Lowering the concentration of profilin in the micropipette to 50 μM slowed the response, as might be expected, but ZmPRO1 and ZmPRO4 were still significantly different from one another ($P = 0.004$), with average nuclear

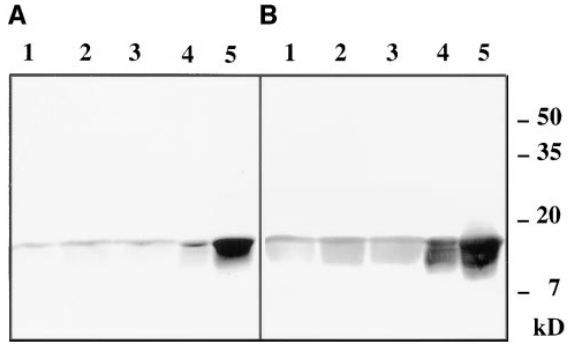

Figure 3. Profilin Can Be Isolated from Developing Endosperm Tissue.

(A) Coomassie blue–stained polyacrylamide gel. Lane 1 contains PLP-purified profilin from endosperm collected 20 days after pollination; lane 2, PLP-purified profilin from endosperm 16 days after pollination; lane 3, PLP-purified profilin from endosperm 12 days after pollination; lane 4, 2 μg of purified, recombinant ZmPRO4; and lane 5, 10 μg of purified, recombinant ZmPRO4.

(B) Protein gel immunoblot of a gel identical to that shown in **(A)** probed with the anti-maize profilin antiserum.

The positions of the molecular mass standards are shown at right in kilodaltons.

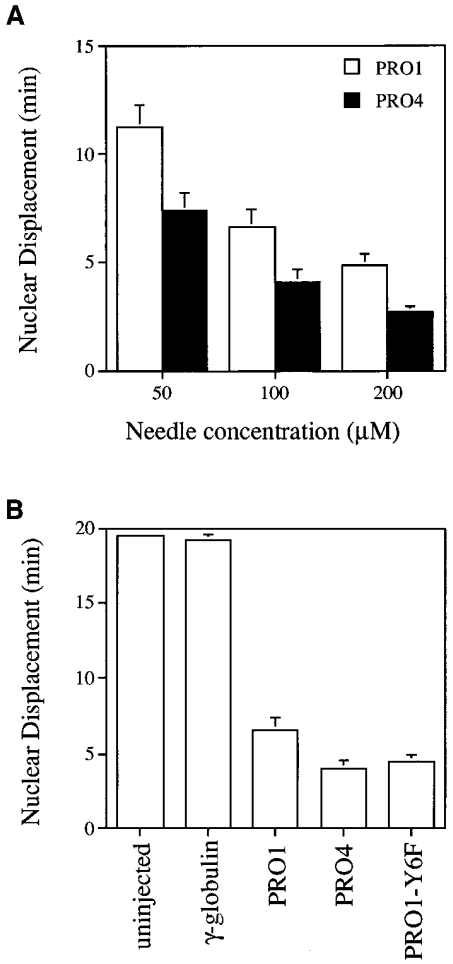

Figure 4. Comparison of the Effects on Nuclear Position of Recombinant Maize Profilins after Microinjection into Stamen Hair Cells.

(A) Dose-dependent effects of excess profilin on cytoplasmic architecture and nuclear displacement were documented in a 20-min microinjection assay. The average nuclear displacement times were plotted for the two recombinant profilin isoforms ZmPRO1 and ZmPRO4 at three different needle concentrations—50, 100, and 200 μM. Error bars represent the standard error, and at least 32 injections were performed at each needle concentration.

(B) Comparison of microinjection of 100 μM needle concentration of ZmPRO1, ZmPRO4, and ZmPRO1-Y6F. Uninjected cells were not penetrated with a needle, and 1.4 mg/mL γ-globulin was microinjected to control for the effects of introducing foreign proteins into the cytoplasm.

displacement values of 11.3 ± 1.0 min (n = 38) and 7.4 ± 0.8 min (n = 35), respectively.

Characterization of PLP and G-Actin Binding Properties

To understand the biochemical basis for the rapid effect of ZmPRO4 on nuclear position in the living cell, we determined equilibrium dissociation constant (K_d) values for binding to PLP and G-actin. The affinity of profilin for PLP was determined by monitoring the enhancement of intrinsic tryptophan fluorescence that occurs upon profilin binding to PLP. The relative fluorescence change was plotted against the concentration of PLP, and the K_d value was derived by nonlinear least squares regression of the data fit to a hyperbolic function. The average values are shown in Table 1. The average K_d value for ZmPRO4 binding to PLP, as determined from six experiments, was 173 ± 17 μM proline residues (mean ±SD; Table 1). This value was significantly lower ($P < 0.002$) than the K_d values of ~275 μM for all of the other maize pollen profilins reported previously (Gibbon et al., 1997). Because profilin binds with highest affinity to oligomers of ⩾10 proline residues (Perelroizen et al., 1994), the values stated in micromolar concentrations of proline residues represent a 10-fold higher apparent K_d than that for a decamer. Thus, the measured K_d of 173 μM proline residues would represent an apparent K_d of 17.3 μM proline decamer. We have reported the values in μM proline residues because we are adding high molecular weight oligomers of mixed length and to allow direct comparison with previous studies (Perelroizen et al., 1994; Petrella et al., 1996; Gibbon et al., 1997).

The binding of profilin to Mg-ATP–actin and Ca-ATP–actin from maize pollen was measured by titrating solutions of G-actin with profilin and monitoring changes in intrinsic tryptophan fluorescence. When the change in fluorescence was plotted against the concentration of profilin, in the presence of actin the total fluorescence was less than would be expected for the additional protein added to the cuvette. The plots were used to derive K_d values, and Table 2 shows a summary of the average K_d values obtained from several experiments. The average K_d for ZmPRO4 binding to Ca-ATP–actin was 2.4 ± 1.7 μM (mean ±SD; n = 3). Similar experiments performed using Mg-ATP–actin yielded an average K_d of 2.3 ± 2.1 μM (n = 3) that was nearly identical to Ca-ATP–actin (Table 2). The affinities of ZmPRO1 and native pollen profilin for Mg-ATP–actin were also determined, and the average K_d values were 1.2 ± 0.6 μM (n = 6) and 1.1 ± 0.7 μM (n = 3), respectively (Table 2). There were no significant differences ($P > 0.05$) observed between the profilin isoforms binding to either Mg-ATP–actin or Ca-ATP–actin. Likewise, no significant differences in the affinity for Mg-ATP–actin were detected between any pairwise combination of profilin isoforms. To test for possible differences in the affinity of either ZmPRO1 or ZmPRO4 for other actin isoforms, the K_d values for binding to human platelet Mg-ATP–actin were also determined and found to be 1.1 ± 0.3 μM (n = 3) and 2.3 ± 0.9 μM (n = 3), respectively. These values were not significantly different by the t test ($P > 0.05$), nor were they significantly different from the values obtained in experiments measuring the association with pollen actin.

The affinity of ZmPRO1 and ZmPRO4 for pollen actin was also tested under polymerizing conditions by measuring the shift in critical concentration (C_c) at steady state in the presence of profilin. Figure 5 shows a representative experiment. The K_d determined from these data for ZmPRO1 was 2.5 μM and for ZmPRO4 was 2.4 μM. The average K_d values measured for multiple preparations of ZmPRO1 and ZmPRO4 were 1.6 ± 0.7 μM (n = 4) and 1.9 ± 0.9 μM (n = 5), respectively (Table 2). These values were not significantly different

Table 1. Affinity of Maize Profilins for PLP

Protein	K_d (μM Proline Residues) Mean ±SD (n)[a]
ZmPRO1	275 ± 15 (4)[b]
ZmPRO4	173 ± 17 (6)
ZmPRO1-Y6F	130 ± 24 (8)

[a] The K_d value for each recombinant profilin isoform binding to PLP was measured by enhancement of intrinsic tryptophan fluorescence. All of the profilin isoforms were significantly different from each other by the two-tailed t test ($P > 0.05$).
[b] Denotes data from Gibbon et al. (1997).

Table 2. Affinity of Maize Profilins for Ca-ATP–Actin and Mg-ATP–Actin

	Intrinsic Tryptophan Fluorescence[a]		
Protein	K_d (μM) Ca-ATP–Actin Mean ±SD (n)	K_d (μM) Mg-ATP–Actin Mean ±SD (n)	Steady State[b] K_d (μM) Mean ±SD (n)
Native	1.4 ± 0.6 (4)[c]	1.1 ± 0.7 (3)	ND[d]
ZmPRO4	2.4 ± 1.7 (3)	2.3 ± 2.1 (3)	1.9 ± 0.9 (5)
ZmPRO1	1.0 ± 0.3 (4)[c]	1.2 ± 0.6 (6)	1.6 ± 0.7 (4)
ZmPRO1-Y6F	ND	0.9 ± 0.4 (6)	ND

[a] The K_d values of each profilin isoform for maize pollen G-actin in either the Ca-ATP– or Mg-ATP–bound state were measured by monnitoring changes in intrinsic tryptophan fluorescence.
[b] The interaction of profilin with actin was also measured under polymerizing conditions by determining the apparent shift in C_c at steady state in the presence of 2 μM profilin. A value for K_d was derived using the equation stated in Methods. No statistically significant differences were found between any of the isoforms using the two-tailed t test.
[c] Denotes data from Gibbon et al. (1997).
[d] ND, not determined.

by the two-tailed *t* test ($P > 0.05$). The average C_c for maize pollen actin, using data from all experiments reported here, was 0.4 ± 0.1 μM ($n = 5$). This value is somewhat higher than that for rabbit skeletal muscle actin, which has a C_c of ~0.2 μM (Sheterline et al., 1995).

The ZmPRO1-Y6F Mutant Shows Gain of Function for PLP Binding

To examine whether the ability of ZmPRO4 to disrupt cytoarchitecture was due principally to its PLP binding properties, a mutational analysis of conserved residues implicated in profilin's ability to bind oligoproline sequences (see Mahoney et al., 1997, and references therein) was performed. A gain-of-function mutant with a single amino acid substitution, ZmPRO1-Y6F, was created, and its properties were compared with the wild-type profilin isoforms. The average K_d for ZmPRO1-Y6F binding to pollen Mg-ATP–actin was 0.9 ± 0.4 μM (mean ±SD; $n = 6$) and was not significantly different from either wild-type profilin isoform ($P > 0.05$). The mutant profilin had a substantially lower K_d for PLP, 130 ± 24 μM proline residues (mean ±SD; $n = 8$), than did the wild-type ZmPRO1, and it was found to be significantly different by the *t* test ($P < 0.0001$). The mutant profilin was microinjected into stamen hair cells to determine whether the increased affinity for PLP correlated with an enhanced ability to alter cytoarchitecture. The mutant profilin at 100 μM tip concentration was as effective as ZmPRO4 at perturbing cytoarchitecture, causing displacement in 4.5 ± 0.4 min (mean ±SE; $n = 42$; Figure 4B), and was significantly faster than wild-type ZmPRO1 by the *t* test ($P = 0.02$).

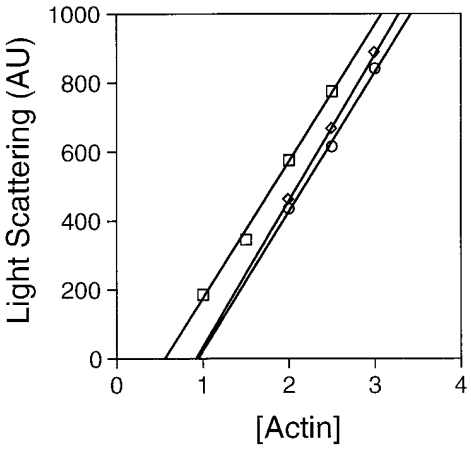

Figure 5. Analysis of the Binding of Recombinant Maize Profilin to Pollen Actin.

The extent of actin polymerization at steady state was measured by 90° light scattering in the absence of added profilin (box) or in the presence of 2 μM ZmPRO1 (diamond) or 2 μM ZmPRO4 (circle). The C_c (x-intercept) calculated for actin alone was 0.57 μM; in the presence of 2 μM ZmPRO1 or ZmPRO4, the C_c was 0.94 and 0.95 μM, respectively. The K_d value calculated using these C_c values and the equation stated in Methods was 2.5 μM for ZmPRO1 and 2.4 μM for ZmPRO4. AU, arbitrary unit.

DISCUSSION

We report the biochemical characterization of a novel profilin isoform from maize, ZmPRO4. The recombinant protein is indistinguishable from other maize profilin isoforms in its affinity for maize pollen actin, but it has a significantly higher affinity for PLP. Furthermore, this new profilin isoform disrupts cellular architecture more rapidly than does any other maize profilin. A gain-of-function mutant, ZmPRO1-Y6F, was also more effective in the living cell and had higher affinity for PLP, supporting the observation that PLP binding properties correlate with cellular function. Our previous work demonstrated functional differences between vertebrate and pollen profilins in an actin-dependent nuclear position assay as well as in their affinity for Ca-ATP–actin from pollen (Gibbon et al., 1997). However, in this study, we demonstrate that profilin isoforms expressed in one cell type can have measurable functional differences in living cells and that the observed differences may be due to binding proline-rich sequences rather than to differences in binding to G-actin.

Many organisms, including vertebrates, Dictyostelium, and Acanthamoeba, have two or three profilin isoforms. The profilin isoforms expressed in these organisms differ in their affinity for G-actin (Haugwitz et al., 1991; Gieselmann et al., 1995), polyphosphoinositides (Machesky et al., 1990; Lambrechts et al., 1997), or PLP (Lambrechts et al., 1997). Despite detailed knowledge about differences in affinities for ligands in vitro, a potential role for these differences in regulation of the actin cytoskeleton or cellular function in vivo is unclear. The Acanthamoeba profilin isoforms recently have been shown to have unique subcellular distributions (Bubb et al., 1998). The isoform with low affinity for polyphosphoinositides is found primarily in the cortical cytosol and codistributes with G-actin, whereas the isoform with high affinity is concentrated in filopodia and microspikes. It has been suggested that the two bovine profilin isoforms have complementary affinities for PLP and polyphosphoinositides: profilin I binds to PLP poorly and polyphosphoinositides with high affinity, whereas profilin II binds to PLP with high affinity and to polyphosphoinositides poorly (Lambrechts et al., 1997); however, the data supporting this assertion are qualitative. It has been shown by more rigorous methods that the human profilin isoforms I and II have similar affinities for polyphosphoinositides (Gieselmann et al., 1995), but any difference in affinity for PLP between the human profilin isoforms remains uncharacterized. Mutagenesis of human profilin I showed that changes in the conserved residues involved in PLP binding could also affect the affinity for actin and polyphosphoinositides, but the possibility that these

mutations affected the overall conformation of the helices containing the mutations could not be ruled out (Björkegren-Sjøgren et al., 1997). Overall, the significance of biochemically distinct profilin isoforms to cellular function is not well characterized.

Unlike most other organisms, in higher plants there are large multigene families for profilin and actin (reviewed in Meagher, 1991; Staiger et al., 1997). Maize is predicted to have at least five profilin genes (Staiger et al., 1993; this study) and six to eight actin isoforms (Shah et al., 1983; Hightower and Meagher, 1986), but the implications of this diversity have not been addressed. Pollen is of particular interest because at least four profilin isoforms are known to be expressed in this tricellular tissue. We have begun to characterize the properties of each maize profilin in vitro and in living cells to determine whether the multiple isoforms present in pollen are functionally redundant or whether they perform specific functions (Gibbon et al., 1997).

The most notable biochemical difference between ZmPRO4 and the other pollen profilins is its affinity for PLP. Whereas all of the maize pollen profilin isoforms have K_d values of ~275 μM proline residues (Gibbon et al., 1997), ZmPRO4 has a substantially higher affinity, with a K_d of 173 μM proline residues. Eight of the most highly conserved residues in eukaryotic profilins (Thorn et al., 1997), marked with asterisks in Figure 1, have been implicated in PLP binding by using mutagenesis (Björkegren et al., 1993; Haarer et al., 1993), nuclear magnetic resonance spectroscopy (Metzler et al., 1994; Domke et al., 1997), and x-ray crystallography (Mahoney et al., 1997). The hydroxyl group of tyrosine-6 was shown to interact directly with the backbone of the PLP peptide (Mahoney et al., 1997), and the Y6F mutation in human profilin I was shown to elute from PLP–agarose at a lower concentration of urea than did the wild-type protein (Sohn et al., 1995). Therefore, the observation that the ZmPRO1-Y6F mutant has higher affinity for PLP is somewhat surprising. It could be that removal of the hydroxyl group allows for greater hydrophobic interaction in the binding pocket while making the protein more sensitive to denaturation by urea. Analysis of the ZmPRO4 sequence shows that these eight residues are conserved, and comparison to other plant profilin sequences with similar affinity (birch pollen profilin; data not shown) or lower affinity for PLP (ZmPRO1, 2, and 3) revealed no obvious substitutions that could explain the observed differences in PLP binding. The significance of highly conserved residues involved in PLP binding is not known, and the proteins that bind to profilin via proline-rich sequences are just beginning to be discovered and characterized (Frazier and Field, 1997).

The PLP binding activity of profilin has been hypothesized to promote localization of profilin within the cell through interactions with two classes of proline-rich proteins (reviewed in Frazier and Field, 1997). Profilin in animals and yeast interacts and/or colocalizes with several proteins that contain proline-rich, formin homology domains (Manseau et al., 1996; Evangelista et al., 1997; Imamura et al., 1997; Watanabe et al., 1997), but the impact of this association on profilin function or organization of the actin cytoskeleton is not known. Formin homology proteins also interact with small GTPases, and similar GTPases may be involved in the regulation of tip growth in pollen (Lin and Yang, 1997). Mammalian profilin colocalizes with the vasodilator-stimulated phosphoprotein (VASP) (Reinhard et al., 1995), and this association is proposed to promote localization of profilin at sites of actin polymerization. Birch profilin expressed in mammalian cells has a distribution similar to the endogenous profilin (Rothkegel et al., 1996), demonstrating that plant profilin can be localized by similar mechanisms. At present, neither VASP-like nor formin homology domain proteins have been identified in plants, and the search for similar profilin binding proteins would contribute significantly to understanding actin dynamics in plant cells.

The enhanced PLP binding affinity of ZmPRO4 could have important consequences for its localization and/or function in living cells, specifically in developing endosperm or pollen. The presence of profilin in developing endosperm is consistent with a role in the regulation of cytoskeletal dynamics. Profilin can be isolated from endosperm extracts at 12 to 20 days after pollination; by this time, starch granule and protein body formation have begun (Kiesselbach, 1949; Clore et al., 1996). The actin cytoskeleton also rearranges ~12 days after pollination, shifting from a mostly cortical and perinuclear distribution to an abundance of cytoplasmic microfilaments (Clore et al., 1996) that eventually surround the protein storage bodies (Abe et al., 1991). If this redistribution requires filament disassembly and assembly, profilin could serve as a key transducer of these rearrangements.

When the affinities of ZmPRO1 and ZmPRO4 for Ca-ATP–pollen actin versus Mg-ATP–pollen actin were compared, no differences were detected in low ionic strength buffer (Table 2). The same was true for ZmPRO1 and native pollen profilin under low ionic strength conditions. Similar results, using the same assay, have been published for pig spleen profilin and rabbit skeletal muscle actin (Perelroizen et al., 1994). Therefore, it appears that profilin binding to G-actin is relatively insensitive to the cation bound to actin. However, the ability of both vertebrate and plant profilin to enhance polymerization of skeletal muscle actin requires Mg-ATP–bound actin (Perelroizen et al., 1996). Further studies that tested the ability of different maize pollen profilin isoforms to alter polymerization of pollen actin also showed no difference in actin binding activity.

Although ZmPRO4 was the maize profilin most effective at causing nuclear displacement in living *T. virginiana* stamen hair cells, its affinity for actin in vitro was similar to or slightly worse than that of the other pollen profilin isoforms (Gibbon et al., 1997). The similar affinity for actin is not surprising because all of the residues that are proposed to be in direct contact with actin are identical in all of the maize profilins (Thorn et al., 1997). The time required for ZmPRO4 to cause nuclear displacement was approximately the same as that measured for human profilin (Gibbon et al., 1997), yet

ZmPRO4 has an affinity for pollen G-actin that is 10-fold lower (2.4 versus 0.2 μM; Gibbon et al., 1997). Coinjection of ZmPRO4 with maize pollen actin into stamen hair cells ameliorates the effect of profilin on nuclear positioning (Ren et al., 1997), suggesting that the actin binding activity of profilin is responsible for the displacement of the nucleus. The paradox of these results has not yet been clarified.

Several possibilities for the potency of ZmPRO4 in living cells should be considered. First, ZmPRO4 may have a higher affinity for the particular actin isoform(s) present in *T. virginiana* stamen hair cells. If ZmPRO4 has a higher affinity for non-pollen actin isoforms, then measurements of binding to pollen G-actin would not distinguish between the profilin isoforms. This possibility seems somewhat unlikely because binding of pollen profilin isoforms to human platelet actin was similar to binding pollen actin. Perhaps a better experiment would be to test binding to actin from another plant tissue. However, no other reliable source of purified, polymerization-competent plant actin has been reported. A second possibility is that association of profilin with proline-rich proteins might enhance the affinity of ZmPRO4 for G-actin, thereby increasing the rate of actin depolymerization. This is consistent with the higher affinity of ZmPRO4 and ZmPRO1-Y6F for PLP in vitro. However, there are currently no data demonstrating that association with proline-rich proteins can alter the actin binding properties of profilin. Finally, the higher affinity of ZmPRO4 or ZmPRO1-Y6F for PLP could displace endogenous *T. virginiana* profilin from sites where it is sequestered from interacting with G-actin. If the endogenous profilin isoform(s) also had a higher affinity for the endogenous actin, then nuclear position might be more rapidly affected.

The enhanced ability of ZmPRO4 to alter cytoarchitecture in live cells may have important consequences for the mechanism of profilin action during pollen development or in the growing pollen tube. Currently available data demonstrate that profilin is uniformly distributed throughout the vegetative cell and pollen tube (Grote et al., 1993, 1995; Hess et al., 1995; Vidali and Hepler, 1997). However, the possibility that specific components of the heterogeneous profilin population may not be detected without isoform-specific probes has not been adequately addressed. From our RNA gel blot and reverse transcription–PCR analyses, it appears that the *ZmPRO4* transcript is less abundant in pollen than *ZmPRO1* and probably represents a small fraction of the total profilin pool. Yet, if a particular, albeit minor, isoform were targeted to specific sites, it is possible that it could have dramatic local effects on actin dynamics. In support of this theory, profilin isoforms in chemically fixed anthers containing developing pollen of birch and maize (M. von Witsch, F. Baluska, C.J. Staiger, and D. Volkmann, manuscript in preparation) and cryofixed rice microspores (B.Q. Huang, S.Y. Zee, and C.J. Staiger, manuscript in preparation) have been shown to have distinct subcellular distributions when visualized with antibodies that have a differential affinity for subsets of the profilin isoforms.

The presence of an additional profilin isoform expressed in maize pollen with unique biochemical properties and potent ability to alter actin organization in live cells raises many exciting questions. Foremost is whether ZmPRO4 in fact plays a unique role during pollen development and morphogenesis. Reverse genetic approaches are necessary to investigate the role of specific profilin isoforms during pollen germination and pollen tube growth.

METHODS

Nucleotide Sequence Analysis

A cDNA containing the coding region for *ZmPRO4*, originally identified as an expressed sequence tag (accession number T18287; see Shen et al., 1994), was generously provided by T. Helentjaris (currently at Pioneer HiBred International, Johnston, IA). Both strands of an ~850-bp NotI-SalI insert in the pSPORT vector (Gibco BRL, Gaithersburg, MD) were sequenced using dideoxy chain termination, according to the manufacturer's instructions (Sequenase 2.0; U.S. Biochemical, Cleveland, OH). The sequence data have been submitted to the GenBank database and can be found under accession number AF032370.

RNA Gel Blot Analysis

Gene-specific probes for *ZmPRO1* and *ZmPRO4* were generated by polymerase chain reaction (PCR) amplification of the 3′ untranslated regions with specific primers. For *ZmPRO4,* the region between nucleotides 508 and 793 was amplified using the following primers: 5′-TAAAAGTTCGTCATGTTCTG-3′ and 5′-AGTAAAATGCACTCGTTCCC-3′. For *ZmPRO1*, the region between nucleotides 491 and 790 was amplified as described previously (Staiger et al., 1993). Coding region probes for *ZmPRO1* and *ZmPRO4* were generated from the full-length inserts after digestion of the plasmids with EcoRI, or NotI and SalI, respectively. The PCR products and digested plasmid DNA were separated by agarose gel electrophoresis, and the appropriate fragments were excised and purified by using Qiaex II (Qiagen Inc., Chatsworth, CA) and then labeled with ^{32}P-dCTP by using random oligonucleotide primers and T7 polymerase (Prime-It; Stratagene, La Jolla, CA).

Total RNA was isolated according to previously described methods (Hussey et al., 1990) from the following tissues of maize inbred line A188: a series of male spikelets at different developmental stages; anthers containing mature pollen; mature pollen and pollen germinated in vitro; 4-day-old roots; 7-day-old etiolated shoots; mature leaf; endosperm dissected from kernels 12 days after pollination; 28-days-after-pollination embryos; and 5-cm unfertilized cobs. Ten micrograms of total RNA was separated on glyoxal– or formaldehyde–agarose gels and transferred to nylon membranes. The filters were probed sequentially with *ZmPRO4* gene-specific and coding region probes. Prehybridization was performed at 42°C in a solution of 50% deionized formamide, 5 × SSPE (1 × SSPE is 1.8 M NaCl, 10 mM NaH_2PO_4-H_2O, and 1 mM EDTA), 5 × Denhardt's solution (1 × Denhardt's solution is 0.2 mg/mL each of Ficoll, PVP, and BSA), 1% SDS, and 50 μg/mL poly(A)$^+$ (Sambrook et al., 1989). Hybridization was in the same solution plus ^{32}P-labeled probe overnight at 42°C.

High-stringency washes were performed in 0.1 × SSC and 1% SDS at 65°C. Between successive probings, filters were stripped with 5 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, and 0.1% SDS at 75°C.

PCR Amplification of Profilins from Maize Pollen First-Strand cDNA

Total RNA was isolated from ungerminated pollen of maize inbred line A188 with TRIzol reagent (Gibco BRL), according to the manufacturer's instructions. Poly(A)$^+$ mRNA was isolated with a QuickPrep Micro mRNA purification kit (Pharmacia Biotechnology, Piscataway, NJ) and reverse-transcribed with a first-strand cDNA synthesis kit (Pharmacia Biotechnology). PCR reactions were performed with gene-specific primers for both *ZmPRO4* and *ZmPRO1* (as above) and annealing temperatures of 57 and 50°C, respectively. Increasing dilutions of first-strand maize pollen cDNA were used as template, with purified plasmid DNA for *ZmPRO4* and *ZmPRO1* (this report; Staiger et al., 1993) serving as controls. Restriction with SacI and SstI was performed on gel-purified, gene-specific PCR products.

Expression of Recombinant Profilin in *Escherichia coli*

The coding region plus 3′ untranslated region was amplified by PCR, using $Vent_R$ DNA polymerase (New England Biolabs, Beverly, MA). The 5′ primer (5′-CCCATATGTCGTGGCAGGCGTACG-3′) mimics the first six codons of the open reading frame and introduces an NdeI restriction site (underlined). The 3′ primer was the SP6 promoter primer that hybridizes to plasmid sequences outside the multiple cloning site on the 3′ end of the *ZmPRO4* insert. After PCR amplification, the ~700-bp product was digested with NdeI and BamHI and cloned into the pET-3a vector (Novagen, Madison, WI) digested with the same enzymes. A single amino acid change, tyrosine-6 to phenylalanine (Y6F), was introduced into *ZmPRO1* by creating an A-to-T transversion in the primer used for PCR amplification of the *ZmPRO1* sequence (5′-GCCATATGACGTGGCAGACGT TCGTGG-3′; mutation is underlined). PCR was performed using $Vent_R$ (exo$^-$) DNA polymerase (New England Biolabs), and the PCR products were ligated into pGEM-T (Promega, Madison, WI). Plasmid DNA from transformants was digested with NdeI and EcoRI, and the insert was ligated into pET-23a, as described previously for *ZmPRO1* (Karakesisoglou et al., 1996). The authenticity of the cloned products was confirmed by sequence analysis. Strain BL21(DE3) of *E. coli* was transformed with the recombinant plasmids, and protein expression was induced by the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside. Profilin was purified on poly-L-proline (PLP)–Sepharose, according to previously described methods (Karakesisoglou et al., 1996). Typical yields of purified ZmPRO4 ranged from 2 to 4 mg per liter of bacteria.

Maize Kernel Isolation and Protein Immunoblotting

The presence of profilin in endosperm was confirmed by protein gel immunoblot analysis of purified protein from endosperm at 12, 16, and 20 days after pollination. For each isolation, a 2-g (fresh weight) sample of endosperm tissue was extracted with 5 mL of extraction buffer consisting of 150 mM KCl, 20 mM Tris, and 0.2 mM dithiothreitol, pH 7.5. Protease inhibitors were added at 1:200 dilution from a stock solution (Ren et al., 1997). The tissue was ground under liquid nitrogen with a mortar and pestle. Cellular debris was removed by centrifugation at 2800*g* for 5 min, and the supernatant was further clarified at 12,000*g* for 30 min. The clarified extract was supplemented with a 1:200 addition of protease inhibitor cocktail and incubated overnight with PLP–Sepharose beads at 4°C and constant shaking. The PLP–Sepharose beads were collected by centrifugation and washed three times with extraction buffer and three times with extraction buffer plus 2 M urea. The washed beads were boiled for 5 min in protein sample buffer, and the bound proteins were separated by PAGE and blotted onto a nitrocellulose membrane (Schleicher & Schuell). Filters were probed with a rabbit polyclonal serum raised against recombinant ZmPRO3, incubated with a horseradish peroxidase–conjugated secondary antibody (Sigma), and developed as described previously (Karakesisoglou et al., 1996).

Nuclear Displacement Assay

Stamen hair cells collected from freshly opened flowers of *Tradescantia virginiana* were microinjected as described previously (Staiger et al., 1994; Karakesisoglou et al., 1996). The volume injected was held constant by front-loading the micropipette to a measured distance. The injectate volume was estimated to be 5 to 6 pL by loading an equivalent amount of oil into the cytoplasm and calculating the volume of the droplet (Gibbon et al., 1997). At least three separate preparations of each protein were used for all data in this report. Microinjection of buffer alone or 1.4 mg/mL bovine γ-globulin (Bio-Rad), equivalent to the total protein concentration of 100 μM profilin, had no effect on nuclear position relative to uninjected control cells (Gibbon et al., 1997; Ren et al., 1997). The data include at least 30 successful injections for each treatment.

PLP and G-Actin Binding

The equilibrium dissociation constant (K_d) values of profilin isoforms for binding to PLP were determined by monitoring the enhancement of intrinsic tryptophan fluorescence (Perelroizen et al., 1994; Petrella et al., 1996; Gibbon et al., 1997). Solutions of 5 μM profilin were titrated with PLP from stock solutions to a final concentration >2000 μM proline residues. After each addition of PLP, fluorescence was monitored on a spectrofluorimeter (model 8000; SLM Instruments Inc., Urbana, IL), with excitation at 292 nm and emission at 335 nm, and the values were corrected for dilution. The fluorescence maximum (F_{max}) was estimated by extrapolation of the regression line to the ordinate of double reciprocal plots of fluorescence change (ΔF) versus [proline residues]. The data were plotted as $\Delta F/F_{max}$ versus [proline residues], and the points were fit to a hyperbolic function by using MacCurveFit (Kevin Raner Software, Mt. Waverly, Australia) to derive a K_d value.

The affinity of profilin for Ca-ATP–actin and Mg-ATP–actin from maize pollen was determined by quenching of intrinsic tryptophan fluorescence (Perelroizen et al., 1994; Gibbon et al., 1997). Maize pollen actin was purified according to the method of Ren et al. (1997) and stored as G-actin at 4°C in dialysis tubing for several days. Quartz cuvettes were loaded with 0, 0.3, or 0.45 μM pollen actin in buffer G (5 mM Tris, 0.2 mM $CaCl_2$, 0.01% [w/v] NaN_3, 0.5 mM DTT, 0.4 mM ATP, pH 8.0), and profilin was added sequentially from stock solutions of 200 to 300 μM in buffer G. For measurements using Mg-ATP–actin, 0.2 mM EGTA and 50 μM $MgCl_2$ were added, and the solution was mixed for 10 min before the experiment. The total increase

in volume after profilin addition was always <1.5%. Fluorescence was recorded with excitation at 295 nm and emission at 330 nm. After each addition of profilin, fluorescence was recorded for ~1 min. The observed fluorescence minus fluorescence of actin alone was plotted against the concentration of profilin at each point. To determine the K_d value at each actin concentration, the resulting plots were fit with the equation $F_{obs} - F_a = F_p + (F_{pa} - F_a - F_p)[PA]$, where F_{obs} is the measured fluorescence; F_a, F_p, and F_{pa} are the intrinsic fluorescence coefficients for actin, profilin, and the profilin–actin complex, respectively; and [PA] is the concentration of the profilin–actin complex (Perelroizen et al., 1994). Data for each series were discarded if one or more curve fits failed to converge on a value for K_d. Similar measurements were performed for ZmPRO1 and ZmPRO4 binding to human platelet actin (Cytoskeleton Inc., Denver, CO).

The affinity of profilin for pollen actin was also determined under polymerizing conditions by measuring the shift in the critical concentration (C_c) at steady state. Actin polymerization was measured by monitoring scattering of 450 nm light at 90° (Cooper and Pollard, 1982). Profilin was added at a concentration of 2 μM to varying concentrations of pollen actin ranging from 1 to 3 μM in a polymerization buffer (5 mM Hepes, 0.5 mM DTT, 0.2 mM ATP, 50 μM $CaCl_2$, 0.1% [w/v] NaN_3, pH 7.0). Basal values of light scattering were recorded for each sample before addition of 100 mM KCl and 5 mM $MgCl_2$ to initiate polymerization. Actin was allowed to polymerize overnight, and polymerization was recorded by measuring light scattering. The C_c value for actin polymerization was determined on plots of the difference between the final and initial light scattering values versus actin concentration; the intercept of the regression line on the ordinate is the C_c. The K_d was calculated using the formula $K_d = ([P][A])/[PA]$, where [P] is the free profilin concentration, [A] is the C_c for actin polymerization in the absence of profilin, and [PA] is the concentration of profilin–actin complex. The value of [PA] was calculated as C_c in the presence of profilin minus the C_c for actin alone, and [P] is equal to the total concentration of profilin minus [PA].

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